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Article

Assessment of *in vitro* and *in vivo* effect of Quercetin 3-Glucoside, Oxyresveratrol and Quercetin *O*-Hexoside against *Leishmania tropica*

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The aim was to scrutinize the *in vivo* and *in vitro* activities against *Leishmania tropica* with compounds of Oxyresveratrol, Quercetin *O*-Hexoside, and Quercetin 3-Glucoside. The *in vitro* outcomes against *Leishmania* were analyzed for 24-48 hours on *L. tropica* KWH23 promastigotes with compounds materials having 50 – 200 µg/mL concentration with negative control and standard drug Amphotericin B. The compounds were analyzed in *L. tropica* infected BALB/c mice against *Leishmania tropica*. The Quercetin 3-Glucoside shows mean inhibition of extracellular promastigotes after 48 hours at 50, 100, 150, 200 µg/mL were 91.02 ± 0.12, 94.50 ± 0.07, 96.15 ± 0.17 and 97.01 ± 0.08 % respectively. In BALB/c mice, the intracellular amastigotes were 91% cured at 200 µg/mL and mean lesion size decreased to 0.41 ± 0.21 mm ($p < 0.01$). The result shows that Quercetin 3-Glucoside possesses significant anti-leishmanial activity.

Keywords: *Leishmania tropica*. Quercetin 3-Glucoside. Oxyresveratrol. Quercetin *O*-Hexoside. Promastigotes. Amastigotes.

INTRODUCTION

Leishmaniasis is an infectious parasitic disease caused by different strains of *Leishmania*. A total of 30 strains of *Leishmania* are discovered, out of which 20 are pathogenic in nature. WHO reported that 12 million people are affected in 98 countries – 05 continent and 1.5 – 2 million people can be affected on yearly basis. *Leishmania tropica* and *Leishmania major* are the main causative strains for Cutaneous Leishmaniasis in Pakistan and neighboring countries. Pentavalent antimonials are the first-line drug therapy against different species of Leishmaniasis but due to severe toxicity, there is a need for New novel therapeutic agent, in order to reduce the adverse effects and launch safe, economical, effective, and potent anti-leishmanial drugs (Iqbal *et al.*, 2017a; Iqbal *et al.*, 2017b). Quercetin 3-Glucoside has been reported as a major flavonoid and active moiety obtained from different natural sources (Zhang *et al.*, 2018; Gao, Wu, Wang, 2013), possess versified pharmacological activities, like antioxidant, anti-inflammatory, hepatoprotective and antiproliferative properties (Lee *et al.*, 2019). Quercetin *O*-Hexoside has been recognized flavonoid which is found in many natural sources (Tabrez *et al.*, 2021; Thirumal, Surya, Kishore, 2012) possess antioxidant, antibacterial, and antifungal properties (Zheng *et al.*, 2017). Oxyresveratrol is a bioactive compound and derivative of resveratrol, obtained from natural sources - possess significant biological properties e.g., anticancer, breast cancer, colon cancer, hepatoprotective, antiallergic, anti-inflammatory, neuroprotective, antihypertensive, antimicrobial, etc, (Zoofishan, Hohmann, Hunyadi, 2018; Li *et al.*, 2019). In this study, the *in vivo* and *in vitro* outcomes against *Leishmania* were analyzed from the compounds Quercetin 3-Glucoside, Oxyresveratrol, and Quercetin *O*-Hexoside.

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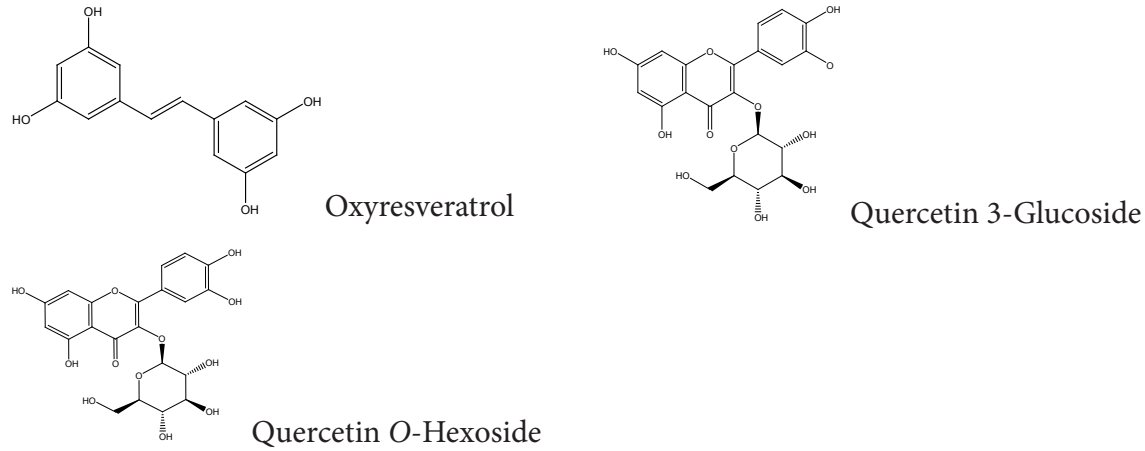

FIGURE 1 - Chemical Structure of Quercetin 3-Glucoside, Oxyresveratrol, Quercetin *O*-Hexoside reported being *Leishmania tropica* inhibitors.

MATERIAL AND METHODS

Chemicals

From Sigma-Aldrich (St. Louis, MO, USA), the chemicals such as RPMI-1640 medium, fetal bovine serum (FBS), formic acid, Quercetin 3-Glucoside (01), Oxyresveratrol (02), Quercetin *O*-Hexoside (03), dimethyl sulfoxide (DMSO), Amphotericin B, penicillin, streptomycin, and analytical grade methanol as a solvent were bought.

In vitro Anti-leishmanial activity

With *Leishmania tropica* metacyclic stationary-phase of promastigotes KWH23, the *in vitro* activity was performed against *Leishmania* of compounds 01, 02, and 03 having concentrations 50, 100, 150, and 200 µg/mL. The inhibition assay for *in vitro* growth against *Leishmania* was acquired from Iqbal *et al.* (2017b). 0.2 mg/mL of streptomycin, 200 U/mL of penicillin, and RPMI-1640 medium containing 10% fetal bovine serum were used for culturing of metacyclic stationary-phase of promastigotes of *L. tropica*. In Gallenkamp incubator (Size 1, UK), at 26 °C for 4 days the parasites were cultivated and after that, harvested in sterile tubes. Hemocytometer (Reichert Technologies, N.Y, U.S.A), measured promastigotes number from 5-10 µL, and below the microscope (CX31, Olympus, Tokyo, Japan) promastigotes number were counted. For live-cell calculation following formula can be used:

$$\text{Live cell count (live cells/mL)} = \frac{Number\ of\ counted\ live\ cells}{Large\ corner\ squares\ number} \times Dilution \times 10{,}000$$

Centrifugation of the harvested promastigotes was done for 10 min 4 °C and 200 rpm, the obtained pellet after removal of supernatant fresh RPMI-1640 medium with 10% FBS were used for reconstitution to acquire 1.5 × 10^6 promastigotes/mL concentration and then poured in a 96 well plate and incubated with compounds 01, 02 and 03 for 2 days at 26 °C (Iqbal *et al.*, 2016). Amphotericin B was the Positive control whereas the DMSO was set as the negative control parameter. Percentage inhibition of parasite growth was calculated as:

$$\text{Percentage inhibition} = \frac{Control\ promastigotes\ count\ -Treated\ promastigotes\ count}{Control\ promastigotes\ count} \times 100$$

In vivo Anti-leishmanial activity

Amson Vaccines and Pharma (Animal center), Islamabad, Pakistan supplied the Male BALB/c mice (20-32 gm; aged 6-8 weeks) which were used. 200 U/ml penicillin, 0.2 mg/mL streptomycin, and RPMI-1640 medium (containing 10% fetal bovine serum) were used

for *Leishmania tropica* KWH23 (1.5×10^6 cells/mL) promastigotes cultivation. In a BOD incubator, the parasite was kept for 4 days where it increases in number at 26 °C and collected. The obtained parasites were kept in sterile tubes and under an upright microscope, it is counted in a hemocytometer. At 4 °C and 200 rpm, centrifugation was done on promastigotes for 10 min. Pellet was obtained by removing the supernatant and was diluted to 10 mL with fresh RPMI-1640 medium containing 10% FBS. It was injected (10 µl) subcutaneously in the right hind footpad (Ozbilgin *et al.*, 2014) while the drug was administrated intraperitoneally (i.p route of drug administration) in BALB/c mice (Rahimi *et al.*, 2021). Utilizing a Dial micrometer the development of lesions was measured weekly during the infection period. After 36 days treatment started due to the establishment and visibility of lesions to the naked eye.

3 groups were treated with compounds, 1 standard drug group, and 1 group was the negative control group total of 5 groups of mice were used. In DMSO the compounds were dissolved and dispensed separately to groups I, II, and III at 4 mg/kg dose for 5 days (each group received one extract only). As a standard drug, Amphotericin B was used at a dose of 15 mg/kg. Group V (negative control) had no drug agent. At 3-day intervals, each mouse received the drug five times and the result was recorded regularly. For weekly checking the deviation of lesion sizes in infected and uninfected mice, a Dial micrometer was used. From infection/lesion areas samples were taken through needle aspirations pre and post-treatment (Iqbal *et al.*, 2017a). Giemsa stain was used under oil immersion for the detection of amastigotes under the light microscope. For the biopsy, from lesion zones, a 60 mg tissue sample was taken on the 48th, 60th, and 90th days post-infection. Under a light microscope, each sample was stained with Giemsa, streaked on slides, and examined.

Statistitical analysis

Nonlinear regression analysis determined the IC50 values using the software Graph Pad Prism 6. Results were taken in triplicate (n = 3) whereas percentage inhibition of parasite was expressed in mean ± SD and $P < 0.05$ was taken as significant.

Moral Record

Department of Pharmacy, The University of Lahore - Islamabad campus (approval ref. no. 038/DOP/UOL) approved this study. The international guidelines on the care and use of laboratory animals, Islamabad Policy, and UOL maintained the animals (The National Academies Press; 2010). During the experiments, the animals were given a standard diet and water.

RESULTS

In vitro Anti-leishmanial test

At 24th h the percentage inhibition of compounds at 50 µg/mL and 200 µg/mL ranged between 30.12 % and 52.30 % and 41.04 % and 64.91 % respectively. The percentage inhibition at 48th h ranged between 67 % and 91.02 % at 50 µg/mL and between 81.12 and 97.01 % at 200 µg/mL (Table I). Against *L. tropica*, the compounds exhibit parasite growth retardation that is 97.01 % inhibition at 200 µg/mL exhibited by compound 01 and 24th h analysis of 02 and 03 shows 41.04 % and 43.01 % at 200 µg/mL in contrast to the negative control.

The significant results against anti-leishmanial activity were shown by compound 01 after 24th h at 200 µg/mL ranging between 52.30 % and 64.9 %.

In vivo Anti-leishmanial test

In the BALB/c mice infused with 0.02 mL *L. tropica* KWH23 (1.5×10^6 promastigotes/mL) effective *in vivo* anti-leishmanial results of compounds 01, 02, and 03 after 36 – 120 days has been shown (Table II). After treatment with the compounds of 3 sample groups at the end of the 8th week, mice average lesion size reduced significantly from 0.81 ± 0.02 mm to 0.41 ± 0.21 mm, but in comparison to the negative control average lesion size was reduced by 1.52 ± 0.1 mm ($p > 0.05$), and of Amphotericin-B group it reduced from 0.84 ± 0.3 mm to 0.38 ± 0.3 mm. The average lesion size after treatment of 8 weeks, and % cure in mice that received compounds 02 and 03 methanolic extracts are 0.52 ± 0.2 mm and 0.48 ± 0.2 mm, respectively; and 73.10 % and 84.21 % respectively.

The *in vivo* anti-leishmanial effects of compound 01 against *L. tropica* shows a decreased average lesion size to 0.41 ± 0.21 mm correlate 91.05 % cure and the result possessed significant activity.

TABLE I - Compounds *in vitro* anti-leishmanial effect

Sample	Sample Concentration (µg/mL)	Promastigotes Inhibition (%)	
		24 h	48 h
01	50	52.30 ± 0.16	91.02 ± 0.12
	100	55.09 ± 0.12	94.50 ± 0.07
	150	61.09 ± 0.56	96.15 ± 0.17
	200	64.91 ± 0.11	97.01 ± 0.08
02	50	30.12 ± 0.01	67.03 ± 0.13
	100	34.76 ± 0.00	73.00 ± 0.14
	150	37.14 ± 0.04	77.00 ± 0.17
	200	41.04 ± 0.18	81.12 ± 0.07
03	50	34.20 ± 0.01	71.09 ± 0.06
	100	35.08 ± 0.02	74.34 ± 0.07
	150	39.19 ± 0.00	79.08 ± 0.03
	200	43.01 ± 0.18	83.45 ± 0.12
Amp	10	61.00 ± 0.80	95.06 ± 0.12
	25	64.16 ± 0.14	96.05 ± 0.15
	50	71.89 ± 0.01	97.28 ± 0.18
	100	73.44 ± 0.03	98.22 ± 0.76
NC	50	00.00 ± 0.00	00.00 ± 0.00
	100	00.00 ± 0.00	00.00 ± 0.00
	150	00.00 ± 0.00	00.00 ± 0.00
	200	00.00 ± 0.00	00.00 ± 0.00

Amp = Amphotericin B, NC = Negative Control.

TABLE II - Compounds *in vivo* anti-leishmanial effect. Table shows the average lesion size in mm ± SD

Sample	Dosage (Duration=5 Days)	Pre-treatment average lesion size (mm)	Post-treatment average lesion size (mm) (After 8 weeks) ($p < 0.01$)	The % Cure rate (confidence interval 95 %)	Cured mice number/ Infected mice number	Average sustenance time (Days)
01	4 mg/Kg	0.81 ± 0.02	0.41 ± 0.21	91.05 (81.76 – 98.06)	6/6	≥ 60
02	4 mg/Kg	0.83 ± 0.21	0.52 ± 0.2	73.10 (61.02 – 86.00)	4/6	≥ 60
03	4 mg/Kg	0.80 ± 0.10	0.48 ± 0.2	84.21 (72.01 – 91.04)	3/6	≥ 60

TABLE II - Compounds *in vivo* anti-leishmanial effect. Table shows the average lesion size in mm ± SD

Sample	Dosage (Duration=5 Days)	Pre-treatment average lesion size (mm)	Post-treatment average lesion size (mm) (After 8 weeks) ($p < 0.01$)	The % Cure rate (confidence interval 95 %)	Cured mice number/ Infected mice number	Average sustenance time (Days)
Amp	15 mg/Kg	0.84 ± 0.3	0.38 ± 0.3	97.53 (81.87 – 97.21)	6/6	≥ 60
NC	4 mg/Kg	0.90 ± 0.33	1.52 ± 0.1	0.00	0/6	≥ 0

Amp = Amphotericin B, NC = Negative Control, Route of Drug Administration = Intraperitoneally (i.p).

DISCUSSION

Quercetin existed with its derivatives especially having glucoside residue in many natural resources, exhibited antioxidant, anti-inflammatory, hepatoprotective, and glutathione depletion properties (Lee *et al.*, 2019; Mehwish *et al.*, 2019). In the gut, bioavailability studies showed that Quercetin glucosides are converted to a glycan via enzymatic reaction, which enhances its absorption when reaching the intestine (Batiha *et al.*, 2020). Oxyresveratrol belongs to the stilbenoid family – structurally similar to resveratrol having a wide variety of pharmacological activities like anticancer, antivirus, antihelminthics, antioxidant, DNA cleavage. These activities may be due to ROS generation in compound 02 which is already reported in various flavonoids (Radapong, Sarker, Ritchie, 2020). Compound 01 at a concentration of 50 µg/mL and 200 µg/mL ($p < 0.01$) after 48h exhibit *in vitro* inhibition ranging between 91.02 ± 0.12 % and 97.01 ± 0.08 % (Table I) which realized that two -OH (hydroxyl group) attached on ring A, along with double bond between C-2 and C-3 proves the inhibition of *Leishmania* strains by compound 01 and 02 while only negative charge oxygen ion makes the compound 01 more potent anti-leishmanial than compound 02 and 03 - Compound 01 has shown marked *in vivo* and *in vitro* effects against *L. tropica* promastigotes in comparison to 02 and 03 which is probably due to the presence of hydroxyl group. After the 8th-week average lesion size was reduced to 0.41 ± 0.12 mm with 01 compound administration and mostly within 120 days BALB/c mice were cured of the infection (Table II), in compliance with previous findings (Iqbal *et al.*, 2017b). Secondary metabolites such as the phenolic group are known which confirmed the presence of anti-leishmanial activity in compound 01 (Iqbal *et al.*, 2016). The compounds 01, 02, and 03 were engaged with the effect of Reactive Oxygen Species (ROS) which showed that it possesses anti-leishmanial activities (Table I and Table II) as reported previously (Cataneo *et al.*, 2019; Radapong, Sarker, Ritchie, 2020). The mechanism involved may be *L. tropica* strains metabolic pathways suppression induced promastigotes/amastigotes death. Microbial enzymes and plant NADH dehydrogenase may be inhibited by the activity of the -OH group (Iqbal *et al.*, 2017a). Cytotoxic effects of compounds 01, 02, and 03 were determined previously on mammalian cell lines which reported that these are safe and non-toxic (Batiha *et al.*, 2020). It is in agreement with the previous findings that flavonoids play a crucial role as anti-leishmanial activity of phytochemical constituents.

This thorough study involves the *in vivo* and *in vitro* effects of compounds 01, 02, and 03 against *L. tropica* parasites are firstly reported while various biological effects of them were known already.

CONCLUSION

Based on *in vitro* and *in vivo* results, it showed that these phytoconstituents inhibited the targeted role of *L. tropica* which are responsible for their survival and growth. As far sequence of anti-leishmanial activity, it is given as; Quercetin 3-Glucoside > Quercetin *O*-Hexoside > Oxyresveratrol. Further studies are required to get detail of the structure-activity relationship and mechanism of

action of the anti-leishmanial compounds. In conclusion, Quercetin 3-Glucoside possess significant anti-leishmanial effect which can be used as a single or in combination with already existing anti-leishmanial agents.

CONFLICTING INTEREST

There is no conflicting interest related to this work declared by the author.

AUTHORS CONTRIBUTION

Accountability of the claim regarding this article content will be borne by the authors and we declare that this work was done by Mr. Kashif Iqbal.

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